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Characterization of the paramagnetic iron-containing redox centres of *Thiosphaera pantotropha* periplasmic nitrate reductase

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Abstract

Electron paramagnetic resonance spectroscopy signals attributable to low-spin haem *c* in the oxidised protein and $[4Fe–4S]^{1+}$ in the dithionite-reduced protein were identified, at low temperature, in *Thiosphaera pantotropha* periplasmic nitrate reductase. Spin integration of these signals as well as elemental analysis suggest a stoichiometry of 1.3–1.6 *c*-haem and 1 [4Fe–4S] cluster per enzyme molecule. The E_m (at pH 7.4) of the $[4Fe–4S]^{2+,1+}$ couple, −160 mV, means that it is unlikely to be physiologically reducible. Peptide sequences from the 90 kDa subunit indicate that the enzyme is a member of the family of molybdopterin guanine dinucleotide-binding polypeptides, the majority of which possess a putative [4Fe–4S] cluster binding sequence and thus may also bind a (low potential) iron–sulphur cluster.

Key words: Periplasmic nitrate reductase; Iron–sulphur protein; cytochrome *c*-type; *Thiosphaera pantotropha*

1. Introduction

A periplasmic nitrate reductase linked to the electron transport chain is found in many species of bacteria. The enzyme from *Thiosphaera pantotropha* has been most extensively characterized [1,2] but the enzyme has also been purified from *Rhodobacter sphaeroides* f.sp. *denitrificans* [3], *Rhodobacter capsulatus* [4–6] and *Alcaligenes eutrophus* [7]. In *T. pantotropha*, the enzyme comprises a 90 kDa molybopterin-binding catalytic subunit (NapA) and a 16 kDa cytochrome-c_{552} (NapB). Mass spectrometry and redox potentiometry suggested that NapB binds two haem groups [1]. In this paper we report the low temperature EPR characterisation of iron-containing centres in *T. pantotropha* periplasmic nitrate reductase.

2. Materials and methods

The periplasmic nitrate reductase was purified from *T. pantotropha* strain M6 (*ΔnarH*::*Tn5*(Km^r) [2]) as previously described [1] except that the final gel filtration step was omitted. Protein concentration was estimated using the Bradford method [8]. Acid-labile sulphide was determined by the Methylene blue method of Fogo and Popowski [9] as modified by Beinert [10] with all operations performed in an anaerobic glovebox (Faircrest Engineering, Croydon, UK) operating under a nitrogen atmosphere (O_2 < 2 ppm). The standard sulphide solution was titrated by iodometry [11]. Iron was determined colorimetrically using Ferene S (Aldrich; 3-(2-pyridyl)-5,6-bis(2-[5-furylsulphonic acid])1,2,4-triazine) [12]. Iron was mobilised from the polypeptide by Lovenberg extraction techniques [13].

EPR spectra were recorded on a Bruker ER-200D X-band Spectrometer (operating frequency 9.4 GHz) equipped with an Oxford instruments ESR-900 helium flow cryostat. Spin integration was performed according to Aasa and Vangard for Kramers doublets signals, using horse met-myoglobin cyanide (3 mM) and CuEDTA (1 mM) as standards [14]. Dithionite reduction of the enzyme was performed in the anaerobic glovebox by incubating the samples with excess dithionite for 15 minutes. Complete reduction was tested by establishing the development of a purple patch, indicative of reduced methylviologen (MV^+), on applying an aliquot of the nitrate reductase sample onto a piece of filter paper impregnated with oxidised methylviologen (MV^{2+}). Mediated redox potentiometry was carried out essentially as described by Dutton [15]. Mediators (20 μM) were Methylene blue, Indigo carmine, anthroquinone-6,6-disulphonate, phenosafranine, benzylviologen, methylviologen and Neutral red. Samples were poised at room temperature then transferred to an EPR tube and frozen in liquid nitrogen. All potentials quoted are with respect to the normal hydrogen electrode.

For amino acid sequence determinations, nitrate reductase preparations were reduced with 2-mercaptoethanol, resolved on a 10% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride-type membrane (ProBlott, Applied Biosystems Ltd., Warrington, UK) [54]. The nitrate reductase NapA polypeptide was excised and digested 'in-situ' with trypsin (sequencing grade from Promega Ltd., Southampton, UK) essentially as decribed in [55], using an enzyme/substrate ratio of 1:20 (w/w). Released peptides were resolved by reverse phase HPLC, using a Brownlee Aquapore RP-300 column (100 × 2 mm, Applied Biosystems Ltd., Warrington, UK) connected to a Severn Analytical HPLC system (HPLC Technology Ltd., Macclesfield, UK). Selected peptides were N-terminally sequenced on an Applied Biosystems 470A protein sequencer with on line PTH analyser.

Amino acid sequences were aligned using the program PILEUP included in the University of Wisconsin Genetic Computer Group package [16] release 7.3. Database searches utilised the BLAST [17] sequence server at NCBI.

3. Results

The 15K EPR spectrum of oxidised (as prepared) *T. pantotropha* periplasmic nitrate reductase is shown in Fig. 1. The rhombic signal, which we have assigned to low spin ferric haem, has $g_z = 2.92$, $g_y = 2.26$ and

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Abbreviations: EPR, electron paramagnetic resonance; MGD, molybdopterin guanine dinucleotide.

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g_x = 1.5. The presence of a small proportion of high spin haem is also indicated by the weak g = 6.0 resonance. The two derivatives at g = 4.3 and g = 2.04 are probably caused by, respectively, adventitiously bound FeIII and CuII. Integration of the $g_z$component of the low-spin haem signal, of two different enzyme preparations, gave intensities corresponding to 1.3 and 1.6 spin/mol (based on an enzyme molecular weight of 110 kDa [1]). In the light of previous results [1], these figures are taken to indicate the presence of two *c*-haems per enzyme. Spectra acquired over a range of redox potentials show that the low-spin haem signal had maximal intensity above +125 mV and could not be detected at −100 mV. Such behaviour is consistent with the reduction potentials of the two *c*-haems determined previously by room temperature visible spectropotentiometry [1].

EPR spectra of the dithionite-reduced enzyme recorded at 18K and two different microwave powers are shown in Fig. 2a and b. The dominant rhombic spectrum (g_1 = 2.03, g_2 = 1.94 and g_3 = 1.89) is very intense and must therefore represent a major paramagnetic species in the sample. Variable temperature studies at 2 mW microwave power showed that this signal could be observed up to 40K and to be almost completely saturated below 10K. The optimal temperature for detecting the signal was 18K. At this temperature the estimated half-saturation power was around 10 mW. The spectra and

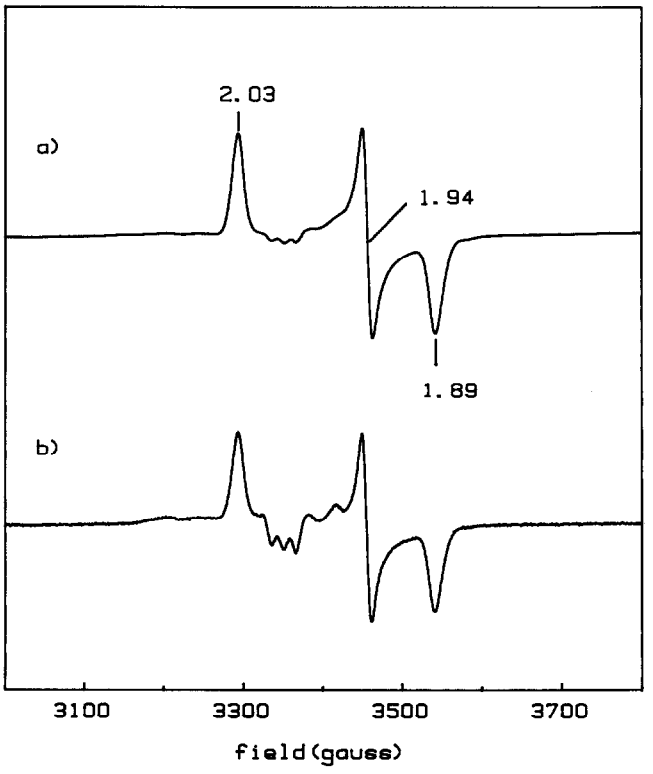

Fig. 2. EPR spectra of dithionite-reduced periplasmic nitrate reductase. Experimental conditions are as for Fig. 1 except that the temperature was 18K and microwave power was (A) 20 mW (gain 5e4) and (B) 0.2 mW (gain 2.5e5).

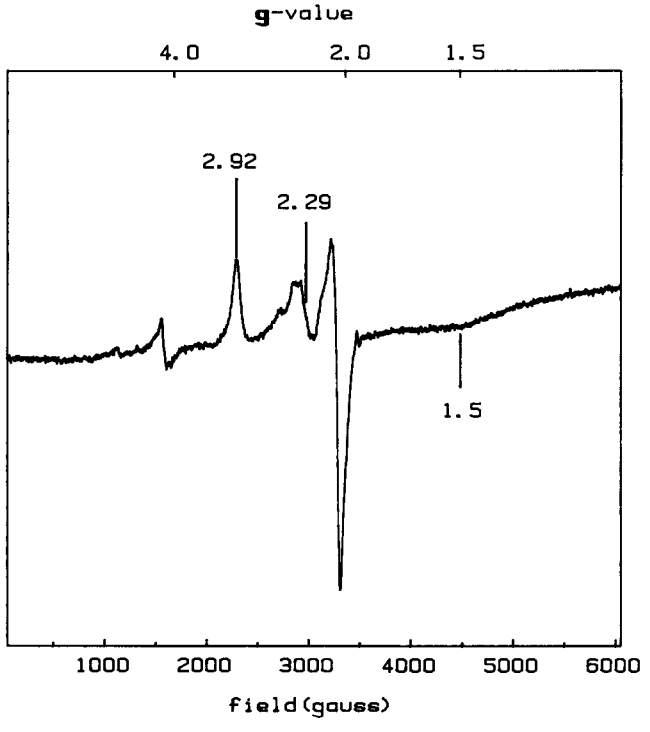

Fig. 1. X-band EPR spectrum of oxidised (as prepared) periplasmic nitrate reductase (10 mg protein/ml) in 30% (v/v) glycerol, 0.1 M HEPES, pH 7.4, 0.1 M NaCl. Experimental conditions: microwave power, 2 mW; temperature, 15.3K; modulation amplitude, 1 mT; microwave frequency, 9.4 GHz; gain, 2.5e5

relaxation characteristics of the major signal are typical of a $[4Fe–4S]^{1+}$ cluster. Accurate spin integration of the $[4Fe–4S]^{1+}$ signal is difficult because of additional signals in the $g \approx 2$ area. These signals are a three-peak signal at g = 2, previously observed with the *E. coli* membrane-bound nitrate reductase and attributed to a 5-coordinate Fe–NO adduct [18–20], and an axial signal ($g\perp$ = 1.98 and $g_{\parallel}$ = 1.95) visible at temperatures up to 150K, which could arise from Mo(V), possibly in a glycerol-inhibited form [21]. Integration of the main 18K signal, in three different enzyme preparations, after correction for the underlying species gave ≈ 1.0 spin/mol enzyme. Mediated redox potentiometry established that the g = 1.89 peak of the iron sulphur signal titrated as a Nernstian n = 1 component with E_m (at pH 7.4) = −160 mV (Fig. 3).

T. pantotropha periplasmic nitrate reductase was previously reported to contain non-haem iron but no acid-labile sulphide [1]. In the light of the EPR experiments we have reexamined the iron and acid-labile sulphide analyses using alternative analytical procedures and employing *Desulphovibrio africanus* ferredoxin I, which contains a single [4Fe–4S] centre [21], as a control. When normalised to the *D. africanus* ferredoxin analysis and taking into account the expected haem iron quantitation (2 mol per mol enzyme, as determined by mass spectrometry [1]) the data in Table 1 indicate that each periplasmic

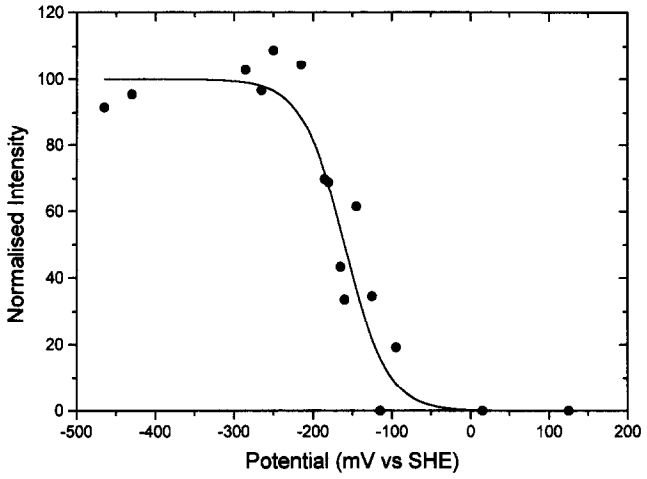

Fig. 3. EPR potentiometric titration of the periplasmic nitrate reductase iron–sulphur cluster. The normalised intensity of the $g = 1.89$ component of the EPR spectrum was plotted versus the measured potential. Data points were collected both from a reductive titration with sodium dithionite and a subsequent reoxidative titration with potassium ferricyanide. The solid line represents a Nernstian $n = 1$ component with $E^{\circ} = -160$ mV. Experimental conditions: 10 mg/ml enzyme in 0.1 M HEPES, 0.1 M NaCl 100 μM EGTA, pH 7.4, 20 μM mediators. EPR parameters: gain normalised to 1e5, temperature, 18K, modulation amplitude, 1 mT, microwave power, 2 mW; microwave frequency, 9.4 GHz.

nitrate reductase molecule contains 4 atoms of non-haem iron and 4 atoms of acid-labile sulphide.

The amino acid sequences of two tryptic peptides derived from the 90kDa subunit of the nitrate reductase have been determined (Fig. 4a). A database search demonstrated that these peptides have highest sequence similarity to the NapA (molybdopterin guanine dinucleotide-binding; MGD) subunit of the periplasmic nitrate reductase of *Alcaligenes eutrophus* and a putative periplasmic nitrate reductase from *Escherichia coli* (Fig. 4a) but no significant sequence similarity to other MGD-binding polypeptides. These similarities indicate, as anticipated [1], that the *T. pantotropha* periplasmic nitrate reductase is structurally related to the *A. eutrophus* enzyme. Near the N-terminus of the *A. eutrophus* [7] and *E. coli* [24] NapA polypeptides is a four cysteine consensus [4Fe–4S] cluster-binding motif [23] which could ligate the iron–sulphur cluster (Fig. 4b).

4. Discussion

The analysis of *T. pantotropha* periplasmic nitrate reductase presented in this paper demonstrates that the enzyme contains an iron–sulphur cluster. This redox centre has not previously been identified in a periplasmic nitrate reductase although the presence of some non-haem iron and a low sensitivity to the iron chelator bathophenathroline had been observed [1,3].

The low temperature EPR spectrum of the iron–sulphur cluster and its relaxation characteristics are typical of a $[4Fe–4S]^{1+}$ cluster although there are examples of $[2Fe–2S]^{1+}$ clusters with similar properties (e.g. in xanthine oxidase [38]). Comparison of the iron and acid-labile sulphide analysis (4 mol iron/4 mol sulphide/1 mol protein) with the spin count (1 spin/1 mol protein) supports the [4Fe–4S] cluster identification as does the presence of a [4Fe–4S] cluster consensus binding motif in NapA from other organisms (Fig. 4b).

The [4Fe–4S] cluster is probably bound by the four cysteine residues at the N-terminus of the NapA polypeptide (Fig. 4b). This motif is conserved in the sequences of most other MGD-binding proteins suggesting that these polypeptides also ligand a [4Fe–4S] cluster (Fig. 4b). While such a cluster has previously been suggested from sequence analysis in some cases [7,27–29,31,36] biochemical evidence for such a cluster is limited to the finding of 3–4 atoms of non-haem iron in FdhF [38] and the possible association of iron sulphur clusters with NarG and DmsA after fragmentation of the enzyme complexes of which they are components [40,41]. An iron–sulphur cluster(s) is clearly present in three prokaryotic assimilatory nitrate reductases [42–44] which are expected to be structurally related to those of known sequence [25,26] (Fig. 4b). The type, number and redox behaviour of the iron–sulphur cluster(s) in these assimilatory nitrate reductases is uncertain. No iron–sulphur centres have been detected biochemically in TorA [45,46] consistent with the absence of the cysteine motif in this enzyme (Fig. 4b). Most interestingly the sequence comparisons shown in Fig. 4b suggest that if an iron–sulphur cluster is liganded by the MGD-binding subunit of the membrane-bound nitrate reductases of *E. coli* then one of the cluster ligands is likely to be a histidine. Such ligation is unprecedented for a [4Fe–4S] cluster

Table 1
Determination of iron and acid-labile sulphide for *T. pantotropha* periplasmic nitrate reductase.

	Iron (mol/mol protein)	Sulphide
Material analysed:		
D. africanus ferredoxin I	3.5	2.7 (1.7)*
T. pantotropha periplasmic nitrate reductase	5.5	4.1 (1.8)*

*For sulphide, the unbracketed numbers are those obtained from samples assayed with internal standards (S^{2-}; standard addition) whilst the bracketed numbers are those obtained by comparison to an external standard curve. The standard addition procedure is more reliable as it takes into account the effect of the polypeptide in the assay system, which tends to lower the S^{2-} recovery efficiency. This efficiency was estimated to be ≈80% for Ferredoxin I and ≈50% for the nitrate reductase.

The data shown for nitrate reductase are for a single enzyme preparation. Analysis of two different nitrate reductase preparations and replicates of the ferredoxin sample indicate errors in the analysis of ±10%

A

		Ref.
T.pantotropha peptide 9213H	GHGHDLAPYDTYHEVR	
A.eutrophus NapA (642-657)	GHGHDLAPFDAYHEAR	[7]
E.coli NapA	GHGHDLAPFDDYHKAR	[24]
T.pantotropha peptide 92-L-009	IIAVPYEPPAEPPDEEY	
A.eutrophus NapA (699-715)	IFALPYEPPAESPDKEY	[7]
E.coli NapA	IFALPFEPAAEAPDEEY	[24]

B

				Ref.
A.eutr.	NapA	(41-97)	KWSKAP C.RFCGTGCG VTVAVKD.....NKVVAT QGDPQAEVNK GLNCVKGYFL SKIMYGQD	[7]
E.coli	NapA		KWDKAP C.RFCGTGCG VLVGTQQ.....GRVVAC QGDPDAPVNR GLNCIKGYFL PKIMYGKD	[24]
K.pneu.	NasA	(1-57)	TETRTT C.PYCGVGCG VIA..... SRAPHGQVSV RGDEQHPANF GRLCVKGAAL GETVGLEG	[25]
Synoc.	NarB	(16-83)	DTAKTL C.PYCGVGCG LEAV....(17).....IWQI RGDRQHPSSQ GMVCVKGATT VAESVSKS	[26]
M.form.	FdhA	(3-59)	KYVPTI C.PYCGVGCG MNLVVKD... ....EKVVGV EPWKRHPVNE GKLCPKGNFC YEIIHRED	[27]
E.coli	FdhF	(1-56)	KKVVTV C.PYCASGCK INLVVDN... ....GKIVRA EA.AQGKTNQ GTLCLKGYYG WDFINDTQ	[28]
W.succ.	FdhA	(54-110)	KKVKTI C.TYCSVGCG IIAEVVD... ....GVWVRQ EVAQDHPISQ GGHCCKGADM IDKARSET	[29]
E.coli	FdoG	(43-106)	RETRNT C.TYCSVGCG LLMYSLGDGA KNAKASIFHI EGDPDHPVNR GALCPKGAGL VDFIHSES	[30]
E.coli	FdnG	(43-106)	KEIRNT C.TYCSVGCG LLMYSLGDGA KNAREAIYHI EGDPDHPVSR GALCPKGPGL LDYVNSEN	[31]
E.coli	NarG	(43-107)	KIVRST HGVNCTGSCS WKIYVKNGLV TWETQQTDYP RTRPDLPNHE PRGCPRGASY SWYLYSAN	[32]
E.coli	NarZ	(43-107)	KIVRST HGVNCTGSCS WKIYVKNGLV TWEIQQTDYP RTRPDLPNHE PRGCPRGASY SWYLYSAN	[33]
E.coli	DmsA	(27-89)	KVIWSA CTVNCGSRCP LRMHVVDG.. EIKYVETDNT GDDNYDGLHQ VRACLRGRSM RRRVYNPD	[34]
B.subt.	OrfX	(2-59)	KVHQSA CPLNCWDSCG FLVTVDDG.. .....KVTKV DGDPNHPITE GKICGRGRML ETKTNSPD	[35]
W.succ.	PsrA	(44-100)	KFVPSI C.EMCTSSCT IEARVEG... ....DKGVFI RGNPKDKSRG GKVCARGSG FNQLYDPQ	[36]
E.coli	TorA	(45-98)	AVISKE GILTGSHWGA IRATVKDG.. ..RFVAAKPF ELDKYPSKMI A.....GL PDHVHNAA	[37]

Fig. 4. (A) Amino acid sequence similarity between two tryptic peptides derived from the 90 kDa subunit of *T. pantotropha* periplasmic nitrate reductase and the NapA subunits of the periplasmic nitrate reductases of *A. eutrophus* and *E. coli*. (B) Alignment of the N-terminal regions of MGD-binding polypeptides showing a possible conserved $[4Fe–4S]^{2+,1+}$-binding motif. *E. coli* NapA is coded by frameshifted reading frames yoj(EDC) at the puative aeg-46.5 locus. NasA/NarB, assimilatory nitrate reductases; FdhA/FdhF/FdhA/FdoG/FdnG, formate dehydrogenases; NarG/NarZ, membrane-bound nitrate reductases; DmsA, membrane-bound dimethylsulphoxide reductase; OrfX, unidentified S- or N-oxide reductase; PsrA, polypsulphide reductase; TorA, periplasmic trimethylamine-*N*-oxide reductase.

but two histidine ligands are indicated for Rieske-type [2Fe–2S] clusters ([47] and references therein). Although the reduction potential of the MGD-subunit iron–sulphur clusters in enzymes other than the periplasmic nitrate reductase is unknown, convincing assignment of both the high potential iron–sulphur centres of the *E. coli* major membrane-bound nitrate reductase to clusters in a subunit other than MGD-binding NarG [19,20,48] suggests that the NarG cluster, like that of NapA, is probably of relatively low reduction potential.

The periplasmic nitrate reductase pathway is linked to the respiratory electron transport chain at the level of the quinol pool [6] (E_m approx +80mV). It is thus highly unlikely that the iron–sulphur cluster (E_m = −160 mV) in the periplasmic nitrate reductase could be reduced by the enzyme's physiological electron donor. Such apparently physiologically irreducible redox centres are now recognised to occur in many electron transport systems e.g. the membrane-bound nitrate and DMSO reductases [19,20,41,48] and the tetrahaem cytochrome *c*subunit of the *Rhodopseudomonas viridis* reaction centre [49]. The function of such centres is obscure. The periplasmic nitrate reductase iron–sulphur cluster identified here might have a purely structural function for which a lack of physiologically accessible redox reactions would be an advantage. The reduction potential of the cluster (−160mV) is, however, considerably higher than clusters thought to have this function in cytoplasmic proteins [50–52]. A regulatory function for the cluster seems unlikely if the cluster is unable to change its reduction state in vivo. We suggest that the cluster may assist in electrons transfer between the *c*-haems in the NapB subunit and the MGD cofactor in NapA, acting not as a centre where an electron is temporarily localised but instead providing a high conductance (highly coupled) Beratan and Onuchic-type pathway [53]. A similar suggestion has been made for the function of the low-potential iron–sulphur centres of *E. coli* membrane-bound nitrate reductase [19].

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